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Experimental CVB3-Induced Chronic Myocarditis in Two Murine Strains: Evidence of Interrelationships Between Virus Replication and Myocardial Damage in Persistent Cardiac Infection

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In order to analyse the relationships between enteroviral replication and the myocardial damage at the onset of chronic cardiac infection, 2 mouse strains with different degrees of immunological competence (NMRI nu/nu, DBA/2) were infected by a myocarditic Coxsackie virus B3 (CVB3-M1) variant. At 31 days post-inoculation, plaque-forming assay, polymerase chain reaction (RT-PCR), and immunohistochemistry were carried out for detecting viruses and viral components in the myocardium. The virological findings were related to histopathological changes in the myocardium as well to the dilatation of both cardiac ventricles. Chronic myocardial lesions characterized by large fibrosis areas and interstitial inflammatory infiltrates were detected together with cardiomegalia in 52.6% (10/19) of athymic mice and in 9% (2/22) of euthymic mice. Viral replication foci were located and were found only in myocarditic cells adjacent to myocardial inflammatory lesions by immunostaining myocardial tissue sections with anti-serum to VP1 virus capsid protein. Using PCR followed by microwell capture hybridization assay, a large excess of viral positive strand RNA over negative strand was semiquantified in heart tissue from mice with chronic myocarditis, whereas approximately equal amounts of plus and minus strand RNA were detected in cases of persistent cardiac infection without chronic myocardial injuries. These findings provide evidence of the major role of viral replication in the pathogenesis of chronic murine CVB3–induced cardiomyopathy. The results indicate that the cardiac persistence of enteroviral RNAs can be observed without chronic cardiomyopathy, which could be explained by a defective viral positive RNA replication. ***J. Med. Virol. 52:206–214, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: CVB3 persistence; semiquantitative RT-PCR; enteroviral replication; dilated cardiomyopathy; chronic myocarditis

INTRODUCTION

Myocarditis is defined as a pathological process characterized by an inflammatory infiltrate of the myocardium with necrosis and/or degeneration of adjacent myocytes [Aretz, 1986]. Enteroviruses of the *Picornaviridae* family, particularly Coxsackie B viruses (CBVs), are considered to be the most common etiological agents of myocarditis. Although most enterovirus illnesses are subclinical, acute myocardial infection can induce severe arrythmias and sudden cardiac death and lead to the development of a chronic form of myocarditis, dilated cardiomyopathy (DCM) [Woodruff, 1980]. Recently, polymerase chain reaction studies in human myocardium have provided evidence that enterovirus infection was detectable in acute and chronic myocarditis as well as in end-stage DCM [Bowles et al., 1986, 1989; Koide et al., 1992; Petitjean et al., 1992; Andréoletti et al., 1996a]. The identity of PCR products amplified from cardiac tissue of patients was established by direct nucleotide sequencing and shown to be related to Coxsackie virus B3 in most of cases tested [Muir et al., 1994]. These studies indicated that Coxsackie B viruses can persist in myocardium through all stages of the progression from acute myocarditis to end-stage congestive heart failure and have a causal role in chronic disease.

Experimentally induced CVB3 chronic cardiac infec-

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tion in various murine models provided evidence for the pathogenesis of human chronic myocarditis [Wolfgram et al., 1985; Huber et al., 1986; Koide et al., 1992; Kandolf et al., 1987, 1993; Sato et al., 1994]. The genetic background, age, gender, and immune status of the mouse as well as the genotype of the virus are known to influence the development of chronic myocarditis [Gauntt et al., 1984, 1988; Khatib et al., 1987; Lodge et al., 1987]. Virus-induced pathobiological events on one hand and different auto-immunological processes triggered by initial viral infection of the heart on the other are assumed to be essential in the pathogenesis of experimental chronic myocarditis, but the exact mechanisms remain to be elucidated further [Chow et al., 1992; Kandolf et al. 1993; Gauntt et al., 1993; Neumann et al., 1994; Sato et al., 1994]. Advances in biological techniques such as the polymerase chain reaction and immunohistochemistry using monoclonal antibodies have provided powerful new tools to define the role of enteroviruses in the chronic phase of myocarditis and to investigate the molecular basis of pathogenicity [Hofschneider et al., 1990; Kandolf et al., 1993].

The interrelationships between virus replication and myocardial damage were studied at the onset of chronic murine CVB3–induced myocardial infection [Kandolf et al., 1993; Klingel et al., 1993]. At 31 days p.i., viruses and several viral components were detected in myocardial tissue from 2 murine models with different genetic backgrounds and degrees of immunological competence. In order to assess the level of genomic viral replication, a quantitative approach of positive and negative CVB3-RNA amounts in heart tissue was carried out using a PCR followed by a microwell capture hybridization assay. All the virological findings were related to histopathological changes in the myocardium as well to the principal morphological feature of dilated cardiomyopathy which is dilatation of both cardiac ventricles. The mechanisms of enteroviral persistence and the relationship with direct viral injury in the pathogenesis of CVB3-induced chronic myocarditis are discussed.

MATERIALS AND METHODS

Viruses

A myocarditic Coxsackie virus B3 variant (CVB3-M1) was produced from the pCVB3-M1 cDNA clone (a gift from Dr. R. Kandolf) by transfection of HeLa cells (ATCC, Rockville, MD) using lipofectin (Gibco, BRL, Paris, France) according to the manufacturer's recommendations [Kandolf et al., 1987]. Viral stocks were purified by isopycnic centrifugation in a CsCl density gradient for 18 hr at 4°C [Sato et al., 1994]. For each viral suspension a microtitration plates methodology was performed and the results were expressed as plaque-forming units (p.f.u.) of virus per ml [Roivanen et al., 1991]. Aliquots of virus stocks were stored at −80°C until use.

Experimental Design

Five-week-old male NMRI nu/nu and DBA/2 mice (Charles River laboratory, Les Oncins, France) were inoculated intraperitoneally with 100 μl of Eagle's minimal essential culture medium (MEM, Gibco) containing 5.10^5 pfu of CVB3. Thirty animals of each strain were divided into 6 groups, including 4 infected mice and 1 control mouse, which was inoculated under the same conditions with 100 μl of sterile MEM. Thirty-one days p.i., all anaesthetized mice were killed by exsanguination. Blood samples were collected on EDTA (10% v/v) into a 1.5 ml Eppendorf tube (Treff, Paris, France), and hearts were removed aseptically. For each infected mouse, the clinical cardiomegaly stage was identified when cardiac ventricle volume was increased to a minimum of 1.5-fold by metric measurement compared to one of the control mice in each group. The ventricle portion was then dissected into 3 pieces which were treated as described previously by Sato et al. [1994].

In Vivo and In Vitro Infectivity of the CVB3 Strains

Heart specimens were homogenized on ice with sterile Eagle's MEM (Gibco BRL, Paris, France) supplemented with 2% fetal bovine serum, 2 mM L-glutamine (Gibco BRL), and gentamycin (50 μg/ml), frozen and thawed 4 times, and cleared of cellular debris by centrifugation (200*g* for 10 min). Supernatants were diluted serially and titrated on Hep2 cells monolayer by a plaque forming assay. Viral titres of heart muscle samples were expressed as a number of pfu per gram of tissue. In order to study in vitro infectivity of the CVB3-isolates, HeLa cell monolayers on 24-well tissue culture plates were inoculated with 10 PFU/cell of CVB3-strains or CVB3-M1 stocks. Duplicate samples were collected sequentially after virus inoculation and titered by the plaque-forming assay as described previously by Arola et al. [1995].

Histopathological Analysis

For each heart sample, four-μm-thick transverse paraffin sections were cut at 3 levels and were examined to detect cellular infiltration, endomyocardial fibrosis, and myocyte necrosis. The severity of inflammatory lesions (cellular infiltrate), necrosis, and fibrosis was graded separately by a pathologist without knowledge of experimental variables using a scale of 0 (no necrosis, inflammation, or fibrosis), 1 (1–10 foci), 2 (11–20 foci), 3 (21–40 foci), and 4 (>40 foci) per section.

Immunohistochemistry

Four-μm-thick paraffin sections of ventricular heart tissue were collected on poly-L-lysine coated slides, deparaffinized with 100% toluene solution, and then rehydrated by graded ethanol solutions. The specimens were incubated with normal goat serum in order to block nonspecific antibody binding (DAKO, Copenhagen, Denmark). Mouse monoclonal anti-enterovirus

antibody solution directed against capsid protein VP1 was used as the primary antibody at a dilution of 1:100 (5D8/1 monoclonal antibody, DAKO, Copenhagen, Denmark). The sections were then washed in PBS and incubated with a 1:20 phosphatase alkaline conjugated anti-mouse globulin (DAKO) [Yousef et al., 1987; Arola et al., 1995]. An alkaline phosphatase development was then performed for 30 min at 37°C with naphtol-AS MX phosphate, levamisol, and Tris-HCl buffer (pH = 7.5). After sections were washed twice in PBS, they were counterstained with haematoxylin-eosin.

RNA Extraction, Reverse Transcription (RT), and Polymerase Chain Reaction (PCR)

Native ribonucleic acid was extracted from frozen myocardial samples by the acid guanidium-phenol-chloroform method as previously described by Chomczynski et al. [1987]. By using either T3 or T7 RNA polymerase (Eurogentec, Angers, France), control positive or negative strand RNA was transcribed from the complete CVB3-cDNA sequence (pCVB3-M1) which was inserted previously in a pBluescript (SK+) vector (Stratagene) as described earlier [Klump et al., 1990]. The amounts of positive and negative RNAs were expressed in OD 260 united, diluted, aliquoted, and then used as external controls in semi-quantitative RT-PCR assays.

RT-PCR assay was carried out as described previously [Andréoletti et al., 1996a]. Two oligonucleotides (sense primer: 5′ CAAGCACTTCTGTTTCCCCGG 3′ and anti-sense primer: 5′ ATTGTCACCATAAGCAGCCA 3′) were selected for an annealing in the 5′ non-coding region (5′NC). In brief, complementary deoxyribonucleic acid (cDNA) for plus or minus strand RNA was synthesized in a total volume of 12.5 μl containing 2.5 μl of the sample, 12.5 pmol of sense or antisense primer, 20 IU Reverse Transcriptase, 20 IU RNAsin, 1 μl of a solution containing 2 mM dATP, dCTP, dGTP, 1.9 mM dTTP and 0.1 mM Dig-dUTP (Boehringer), 2.5 μl RT buffer 10X, and 9.2 μl of sterile water. The reaction was carried out at 37°C for 60 min and was stopped by heating the samples for 5 min at 95°C. Then 37.5 μl of PCR reaction mixture consisting of 5 μl of 10X reaction buffer, 1.25 IU Taq-DNA polymerase, 12.5 pmol of sense or anti-sense primers, 5 μl of dNTPs solution, and 24.4 μl of sterile water were added directly to the cDNA sample. DNA amplification was carried out in a MJ research thermocycler PTC 200 (MJ research, Watertown, MA) for 35 cycles (denaturation, 95°C, 30 sec; annealing, 50°C, 45 sec; extension 72°C, 1 min).

For all myocardial specimens, the glyceraldehyde 3 phosphate dehydrogenase (G3PDH) mRNA was amplified using specific primers (primer sense: 5′ CATGTGGGCCATTTGAGGTCCCACCAC 3′; primer antisense: TGAAGGTCCGAGCAACGGATTTGGT 3′) (Clontech) in a RT-PCR reaction and used as a positive control to prove the absence of Taq-DNA polymerase inhibitors, to verify the RNA extraction and the cDNA transcription. The amplification protocol was the same as above except for annealing at 65°C.

An aliquot of amplified RT-PCR product (15 μl) was subjected to electrophoresis under 100 V in a 2% agarose gel containing 0.5 μl/ml of ethidium bromide.

Southern-Blot Analysis

Southern-blotting procedure was undertaken as described previously by Leparc et al. [1993]. The filters were pre-hybridized at 60°C for 2 hr into the hybridization solution 5 X SSC- 0.1% N-lauroylsarcosine-0.02% SDS-5% blocking solution (provided by Boehringer). The biotin-labelled probe corresponding to a part of the enteroviral 5′NC region was then added to obtain at 15 pmol/ml concentration (EV probe: 5′ b GGCCGCCAACGCAGCC 3′). Hybridization was performed overnight at 45°C. The biotin-labelled hybrids were detected using streptavidin conjugated to alkaline phosphatase (Boehringer). Then the filters were covered with chemiluminescent substrate (CSPD, provided by Boehringer) and exposed on X-ray films (Amersham, Les Ullis, France).

Semiquantitative Detection of Enteroviral Amplicons

A semiquantitative detection of Dig-labelled enteroviral amplicons was carried out using a PCR-ELISA detection system (Boehringer Mannheim, Germany). For each serial assay, a negative substrate blank (buffer blank), a negative control RT-PCR test, and 2 serials of externally amplified standards corresponding respectively to known amounts of positive and negative CVB3-RNA were included. For each myocardial sample, Dig-labelled G3PDH amplicons were detected in a second microwell using a specific DNA capture probe. The microwell hybridization procedure was carried out as described previously by Andréoletti et al. [1996b]. In brief, the hybridization reaction was carried out by adding 5 μl of heat-denatured amplicons and 195 μl of an hybridization solution (provided by Boehringer) containing 15 pmol/ml of a biotin-labelled capture probe (EV probe: 5′bGGCCGCCAACGCAGCC3′; G3PDH probe: 5′ bCAGGGAAGGCCATGCCAGTGA 3′; Genset, France) in a streptavidin-coated test well for 2.5 h at 45°C. After washing the wells 5 times (washing solution buffer provided by Boehringer), enteroviral amplicons labelled with digoxigenin were detected with 200 μl of polyclonal anti-Dig Fab fragments conjugated to peroxidase (Boehringer). Wells were washed 5 times, and then 200 μl of ABTS substrate solution (Boehringer) was added for 30 min at 37°C.

The optical density was measured at 405/492 nm in an ELISA-reader (Behring diagnostic ELISA processor II, France). Results were expressed as net absorbance after the optical density of the substrate blank was substracted automatically for each microwell by computing (ELLA 1.5, Behring Lille, France). For each serial assay, the OD intensities obtained from external standards were plotted against the log RNA copy num-

ber. The quantity of sample positive or negative CVB3-RNA was then calculated by the formula of linear regression. This semiquantification was validated for each sample only if the OD value corresponding to the detection of G3PDH amplicons was higher than 1.5 units.

RESULTS

Clinical and Histological Characteristics

Five nude mice in 3 different groups died spontaneously between days 15 and 18 postinfection. In comparison 2 DBA/2 mice in 2 different groups died at days 14 and at 15 p.i. No death was observed in mock-infected animals.

At 31 days p.i., no control animals had any cardiac histological lesions (Fig. 1A). Within the infected nude mouse group, 53% (10/19) of animals had chronic endomyocardial lesions associated with a cardiomegaly (Table I). The myocardial injury was typically multifocal and characterized by low scores of necrotic lesions, associated with high rates of fibrosis and a few infiltrates of mononuclear cells which provided the basis for the diagnosis of chronic myocarditis (Table II, Fig. 1B). Moreover, 36.8% (7/19) of infected nude mice demonstrated a normal cardiac volume associated with healing lesions (Table II).

Only 9% (2/22) of DBA/2 infected mice developed chronic endomyocardial lesions which were associated with a raising of cardiac ventricles volume (Table I). A semiquantitative analysis of these chronic lesions demonstrated medium scores of necrotic lesions associated with high rates of fibrosis and inflammation, improving the confirmation of the onset of the chronic phase of myocarditis (Table II, Fig. 1C, 1D). Furthermore, 36.4% (8/22) of infected DBA/2 mice showed healing lesions without cardiac dilatation. The histological analysis of these scarring lesions is described in Table II for 2 animals.

Cell Culture

Infectious virus was detectable in endomyocardial tissue from 53% (10/19) of infected athymic mice (Table I). The detection of enteroviral particles in myocardium from nude mice with a mean viral titre of $3.5\ 10^3$ pfu per gram of cardiac tissue was perfectly correlated with the detection of chronic cardiac lesions (Table II). Similarly, only the DBA/2 mice suffering from chronic lesions showed detectable virus particles with a virus titre of $1.5\ 10^2$ pfu per gram of cardiac tissue (Tables I and II). For each CVB3 strain isolated from myocardial tissue, in vitro infectivity was tested to investigate the replication efficiency. One step growth curves using the HeLa cells infected with the CVB3-M1 inoculation variant and cardiac CVB3 isolates showed that the enteroviral persistence was not directly correlated with any detectable in vitro replication characteristics (data not shown).

Immunohistochemistry

The VP1 immunostaining was used to localise viral replication foci in myocardial tissue [Hohenadl et al., 1994]. Heart sections from CVB3-infected mice were positive when focal intensive staining of single cells was observed. The number of detectable immunopositive cells was higher in athymic than in euthymic animals, and staining was localised only to myocarditic cells that surrounded foci of chronic myocardial lesions containing replacement fibrosis and mononuclear cell infiltration (Fig. 1E). A positive VP1 detection was associated with chronic histological lesions and a cardiac dilatation both in nude and DBA/2 murine models (Table I). The heart sections from uninfected control animals, as well as from those treated with BSA instead of primary antibody, were negative.

Polymerase Chain Reaction

RT-PCR assay followed by Southern blotting showed a sensitivity up to 60 genomic CVB3-RNA copies (Fig. 2A). Tissues from uninfected control animals had no viral RNA, nor did the blank controls included in the PCR assays. For each endomyocardial specimen, the glyceraldehyde phosphate dehydrogenase (G3PDH) mRNA was amplified as an internal control to verify removal of PCR inhibitors from RNA extract [Hierholzer et al., 1993] (data not shown). Enteroviral RNA persistence was detectable in 17 of 19 (89.5%) infected NMRI nude mice (Table I). Interestingly, of the 17 mice positive by RT-PCR, 7 were free of viral particles and chronic cardiac lesions (Table II). Of the 22 infected DBA/2 mice, 4 were positive by RT-PCR (Table I); however, only 2 of these animals demonstrated chronic myocarditis lesions associated with infectious viral particles (Table II). Figure 2B shows an example of genomic CVB3-RNA detection in cardiac tissue from DBA/2 and NMRI mice. To determine whether the positive myocardial virus RNA detection could be explained by an enteroviral viraemia, we carried out an RT-PCR assay using 100 μl of whole blood. In both murine models, enterovirus genome was undetectable in blood from controls and infected mice (data not shown). An RT-PCR assay was used to detect minus strand virus RNA in murine myocardium (Fig. 2C). Minus strand RNA was detectable in the cardiac tissue from each mouse demonstrating a genomic enteroviral cardiac persistence. In order to perform a semiquantitative approach of positive and negative RNA amounts in cardiac tissue, we used a colorimetric microwell capture hybridization assay. For each serial assay, externally standards were amplified and the OD values obtained were plotted over the log of the RNA copy number. The curves drawn for the positive and negative CVB3-RNA were similar, showing that the assay yields with these two targets were identical (Fig. 3). The cutoff value was defined as 4 times the OD obtained with the negative controls. The cutoff (0.204 OD units) was below the OD recorded in the assay with 60 copies of plus or minus CVB3-RNA (Fig. 3).

Within the NMRI and DBA/2 mice groups suffering from cardiac dilatation, viral positive strand RNA was present with a mean of 80,00-fold excess over negative strand RNA (Table II). These data were similar to

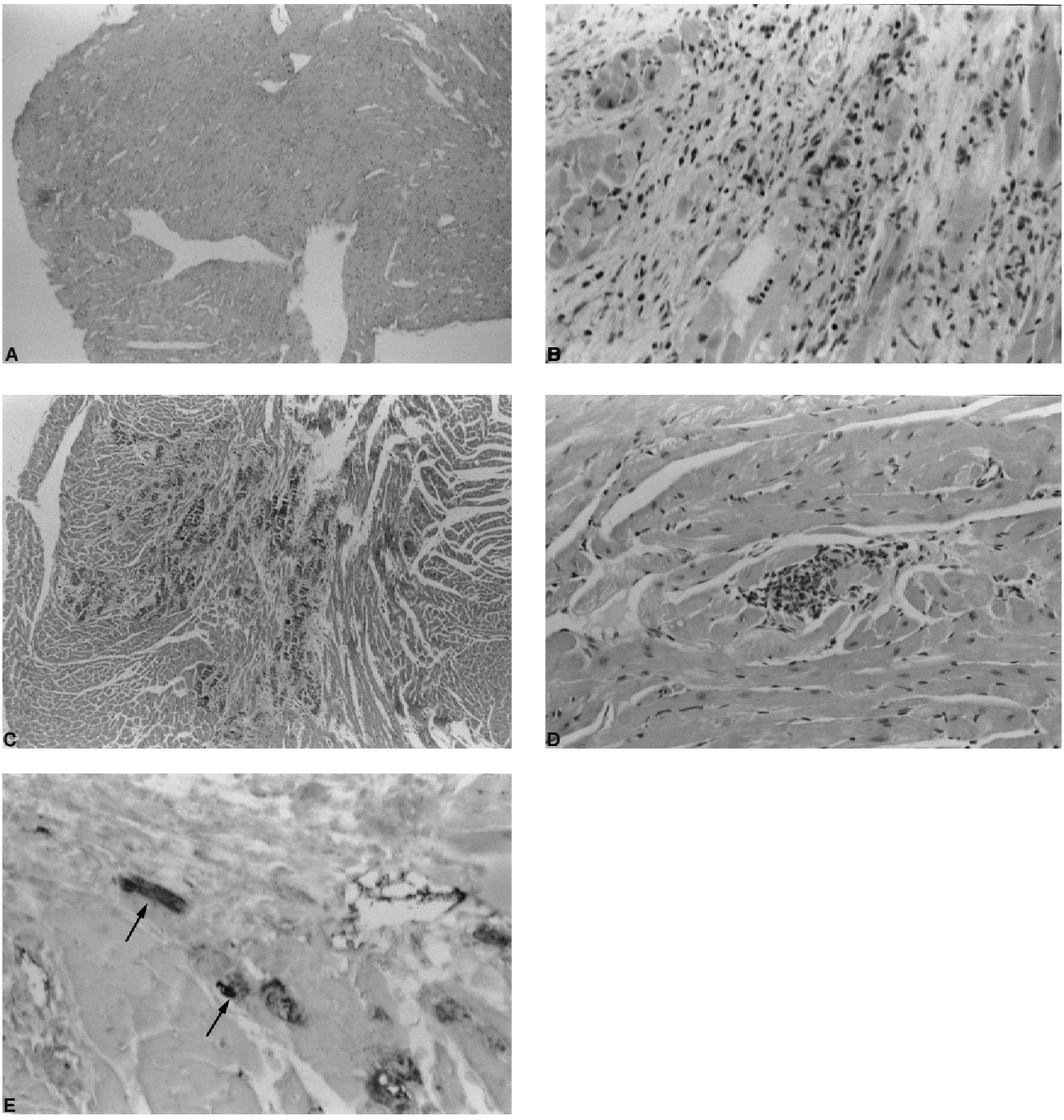

Fig. 1. **A:** Haematoxylin eosin-stained (HES) paraffin-embedded murine heart tissue sections (4 μm) obtained at day 31 p.i. from control nude mice (HES ×25). **B:** In infected nude mice, similar sections (HES ×400) showed replacement of myocytes by a cellular ingrowth of fibroblasts with associated a low mononuclear cell infiltrate. **C:** In DBA/2 mice, chronic endomyocardial lesions were characterized by a focal fibrosis associated with calcifications and inflammatory infiltrates (HES ×100). **D:** In a few cases, DBA/2 mice showed a myofiber necrosis surrounded by polymorphonuclear cells at 31 p.i. **E:** Immunohistological detection of VP1 viral capsid protein into ventricular tissue sections obtained at 31 days p.i.; in myocardial sections, only myocytes (arrows) adjacent to chronic inflammatory lesions were positive for VP1 protein detection.

those obtained from total RNA extracted from CVB3 infected-Hep2 cells at 4 hr p.i. (data not shown). By contrast, NRMRI and DBA/2 mice without cardiac dilatation demonstrated equivalent amounts of plus and minus strands (Table II). In both murine models the mean value of plus and minus RNA amounts in mice without cardiomegaly were approximately similar to the mean amount of minus strand RNA detected in mice demonstrating infectious viral particles. These findings suggested a defective positive RNA replication

TABLE I. Distribution of Virological, Histological, and Clinical Results Between CVB3-Infected NMRI(nu/nu) and DBA/2 Mice at 31 Days P.I.

CVB3-infected mice[a]	Total (n)	Cardiac dilatation[b]	Chronic myocardial lesions[c]	Virus isolation[d]	VP1 antigen[e]	Cardiac CVB3-RNA[f]
NMRI (nu/nu)	19	53% (10/19)	53% (10/19)	53% (10/19)	53% (10/19)	89.5% [g](17/19)
DBA/2	22	9% (2/22)	9% (2/22)	9% (2/22)	9% (2/22)	18% [g](4/22)

[a]Coxsackie virus B3 Nancy strain (CVB3) was inoculated intraperitoneally.
[b]Cardiac dilatation was effective when ventricular volume was increased to a minimum of 1.5-fold compared to uninfected mice.
[c]Histopathological examination: detection of large fibrosis areas associated with inflammatory interstitial infiltrates.
[d]Isolation of CVB3 infectious particles from heart tissue (Hep^2 cell culture).
[e]Endomyocardial detection of VP1 capsid protein by immunostaining.
[f]Endomyocardial detection of virus positive strand RNA using polymerase chain reaction and Southern blotting.
[g]Results for mice with persistent cardiac infection are detailed in Table II.

TABLE II. Results of the Plaque-Forming Assay, Enteroviral PCR, and Histopathology of the Hearts of Mice With Persistent Cardiac Infection at 31 Days P.I.

Mice with CVB3-cardiac infection		Total	Plaque assay[b]	PCR[c]		Histopathology[d]		
Mouse-strains	Subgroups	(n)	Heart (PFU/g)	RNA +	RNA –	Necrosis	Inflammation	Fibrosis
NMRI (nu/nu)	Cardiac dilatation[a]	10	$3.5\ 10^3$	5.17	3.27	0.7	1.7	3.7
	Normal cardiac volume	7	—	3.14	3.09	0	0	0.4
DBA/2	Cardiac dilatation	2	$1.5\ 10^2$	4.53	2.37	1.5	2.5	4
	Normal cardiac volume	2	—	2.24	2.15	0	0	0.8

[a]Cardiac dilatation was effective when ventricular volume was increased to a minimum of 1.5-fold compared to uninfected mice.
[b]The value of the plaque-forming assay are expressed as PFU per gram of myocardial tissue as mean of "n" values; —, no plaques appeared at the level of sensitivity of 100 PFU.
[c]Semi-quantitative detection of plus and minus viral-RNA using PCR followed by colorimetric microwell capture hybridization. The values are expressed as log of RNA copies per gram of myocardial tissue as mean of "n" values.
[d]Myocyte necrosis and inflammation are scored using a scale of 0 (no necrosis, inflammation, or fibrosis), 1 (1–10 foci), 2 (11–20 foci), 3 (21–40 foci), and 4 (>40 foci) per section and expressed as mean number of "n" values.

in cases of persistent cardiac infection without chronic cardiomyopathy (Table II).

DISCUSSION

Chronic CVB3 endomyocardial infections have been described previously in various murine strains with histopathological features similar to heart muscle lesions observed in humans, suggesting that CVB3 persistence in heart was an important factor in chronic myocarditis [Kandolf et al., 1993; Muir et al., 1994]. Enterovirus cardiac persistence as observed in permissive mouse strains with chronic CVB3 myocarditis was found to be associated with a persistent replicating virus, but the molecular mechanisms of enteroviral persistence as well their relationships with direct viral injury in the pathogenesis of CVB3-induced chronic myocarditis remains to be further elucidated [Kandolf et al., 1993; Sato et al., 1994].

We investigated the interrelationships between the enteroviral replication and the myocardial damage at the onset of chronic myocarditis. To this end, 2 murine strains with different genetic backgrounds and degrees of immunological competence were infected by a myocarditic CVB3-M1 Nancy variant obtained by transfection of an infectious cDNA clone [Klump et al., 1990; Klingel et al., 1992]. In order to reach the onset of the chronic phase of myocardial disease which is characterised by myocardial healing lesions containing replacement fibrosis associated with low number of degenerated myocytes and mononuclear cell infiltrates, all mice were killed at 31 p.i. as suggested by the previous studies carried out by Kandolf et al. [1987, 1993]. At this point, 53% of infected NMRI nu/nu mice were suffering from ongoing chronic myocardial lesions associated with a cardiomegaly, while only 9% of infected DBA/2 mice demonstrated the same DCM-features (Table I). In contrast to the chronic myocardial injuries observed at 31 p.i., CVB3-M1 induced acute myocarditis lesions characterised by massive myocyte necrosis and mononuclear cell infiltrates were observed in the myocardial tissue from each NMRI or DBA/2 infected mouse that died during the course of the disease between days 14 and 18 p.i. (data not shown), demonstrating that the acute stage of heart infection was reached approximately 15 days before the chronic stage of the disease in the 2 murine strains [Kandolf et al., 1993]. These experimental data are inconsistent with the results of earlier studies in which chronic myocarditis was a constant finding in NMRI nude mice and was absent in DBA/2 mice [Huber and Lodge, 1986; Kandolf et al., 1987]. These discrepancies could be explained by the replicative characteristics of the CVB3-M1 variant into target tissues, and by subline variations in the genetic background or host response between inbred murine strains [Arola et al., 1995].

A strong correlation between replicative viral particles detection and chronic myocarditis was found in both murine models at 31 p.i. (Table II). These findings, together with previous observations of chronic myocardial lesions in CVB3 mice infected persistently, support the hypothesis that the development of chronic myocarditis is associated with the presence of endomyocardial replicating virus [Klingel et al., 1992, 1993; Sato et al., 1994]. In the present study, viral titres in

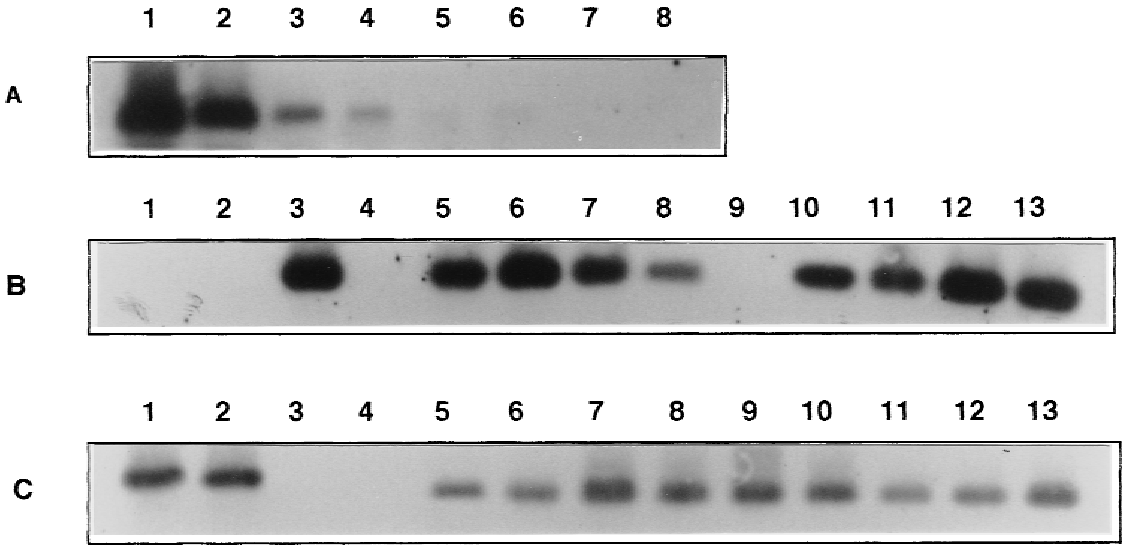

Fig. 2. **A:** Sensitivity of hybridization; the signals were obtained from the hybridization of RT-PCR products derived from the amplification of control positive CVB3-RNA extracted from 10-fold dilutions of a known RNA copy number in 100µl of culture medium, corresponding to 6.10^6 (lane 1) to 6.10^{-2} (lane 8) positive RNA copies. The PCR product was detected in as few as 60 positive RNA copies (lane 6). **B:** Example of CVB3-RNA detection in the myocardium from NMRI nude and DBA/2 mice using RT-PCR followed by Southern blotting. Plus strand RNA was detectable for positive CVB3 control (lane 3), for infected nude mice (lanes 5–8 and lanes 10–11), and for 2 infected DBA/2 mice (lanes 12 and13). No signal was observed in negative RT-PCR controls (lanes 1 and 2), uninfected nude mice (lane 4), or uninfected DBA/2 mice (lane 9). **C:** Specific endomyocardial detection of minus-strand CVB3-RNA by RT-PCR followed by hybridization using biotinylated-probe. Lane 1: minus-strand RNAs transcribed from the complete CVB3-cDNA sequence. Lane 2: total RNA extracted from pCVB3-M1 transfected HeLa cells. Lanes 3 and 4: minus strand RNAs from uninfected NMRI (nu/nu) and DBA/2 mice. Lanes 5 and 6: positive detection of minus strand RNAs in the myocardium from two infected DBA/2 mice. Lanes 7–13: positive detection of minus strand RNAs in the myocardium from infected nude mice.

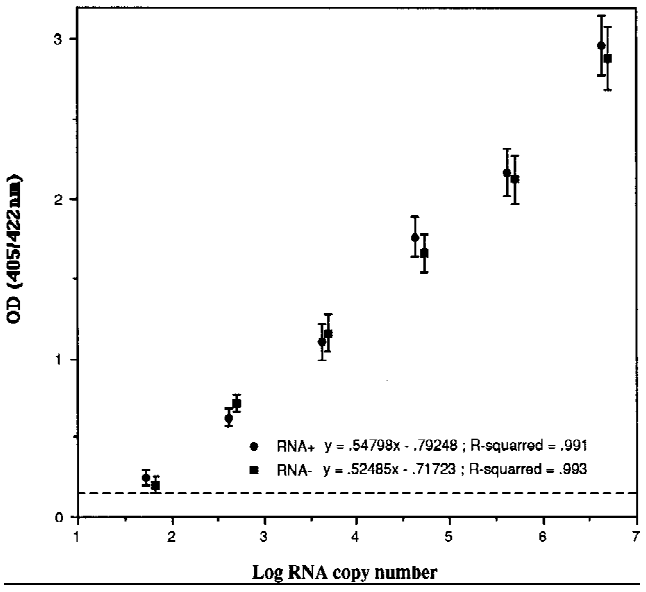

Fig. 3. Results of the externally amplified controls used for semiquantitative EV RT-PCR. The mean values of ODs obtained with either positive or negative RNA were plotted over the log of the RNA copy number. A broken line indicates the cut-off value (OD = 0.204) calculated as 4 times the mean value of the substrate blank and negative RT-PCR controls.

the myocardium were lower in DBA/2 than in NMRI mice and could be explained by the differences of T cell–mediated immunity competence between the 2 murine strains [Chow et al., 1992; Sato et al., 1994].

For each isolated strain, the characteristics of CVB3 replication were studied by one-step growth curves demonstrating no major differences in viral replication efficiency between the viral cardiac isolates and the CVB3-M1 variant (data not shown). These data suggested that the mechanism of endomyocardial CVB3-persistence was not linked to an in vitro detectable modification of virus replication capacity.

In order to localise infection and relate it to the histopathological findings of the specimens, an immunological detection of the VP1 virus capsid protein (Fig. 1E) was undertaken. VP1 protein was detectable in the myocardial tissue from all mice positive by virus isolation. As described previously by Klingel et al. [1992], VP1 positive myocytes were found to be adjacent to ongoing inflammatory lesions, thus providing further support for a spatial correlation between inflammatory myocardial lesions and viral replication at the onset of chronic myocarditis. These findings, together with previous reports [Sato et al., 1994; Arola et al. 1995], are in agreement with the hypothesis of a direct and primary role of virus in sustaining ongoing chronic lesions. Using a RT-PCR assay, positive and negative strand RNA were detectable in the myocardial tissue from each mouse demonstrating ongoing chronic lesions and cardiomegaly. However, we observed in 36.8% of infected nude mice and in 9% of infected DBA/2 mice a plus and minus strand RNA persistence without DCM features (Table II). In cases of cardiac dilatation, semiquantitative RT-PCR results demonstrated a large positive

strand RNA excess over negative strand in agreement with the values obtained from virus RNA synthesis during lytic infection of CVB3-infected cells [Novak et al., 1991; Hellen and Wimmer, 1995]. In contrast to the in vitro situation, enteroviruses associated with persistent non-inflammatory infection of cardiac muscle synthesised approximately equimolar amounts of positive and negative strands of enteroviral RNA. In these cases, the mean amount of positive and negative RNA was similar to the mean amount of minus strand RNA detectable for persistent replicating viruses, suggesting a defective plus strand RNA synthesis (Table II). Cunningham et al. [1990] demonstrated a persistent non-inflammatory enteroviral infection of muscle in patients suffering from chronic fatigue syndrome. In this study the authors demonstrated equivalent amounts of plus and minus strand RNA which could result from a defective control of viral RNA synthesis. In the present report, our data demonstrate for the first time the persistence of a defective virus in non-inflammatory murine myocardial tissues. The semi-quantitative PCR results clearly support the hypothesis of a defective positive RNA-strand synthesis. These findings may explain the detection of low amounts of enteroviral positive RNA by PCR in myocardial tissue taken from patients without any known cardiac pathology or suffering from other cardiac diseases than DCM [Petitjean et al., 1992; Ueno et al., 1995; Andréoletti et al., 1996a]. Therefore, it is possible that immunocompetent subjects without chronic inflammatory cardiac lesions may harbour in the myocardial tissue a non-replicative enterovirus characterized by equivalent amounts of plus and minus RNA. This hypothesis remains to be investigated in further clinical studies by using a quantitative detection of plus and minus enteroviral RNA into ventricular endomyocardial specimens. In the present study, it was observed that a myocarditic CVB3 variant could induce in 2 different murine strains a persistent cardiac infection associated with a complete or defective viral replication. In cases of defective virus, the similar amounts of plus and minus strand RNA could result in inhibition of translation of virus-specific gene products, explaining the failure to assemble infectious progeny virus and the absence of an inflammatory response in the myocardium of several infected mice [Oldstone, 1989]. However, the molecular basis of the mechanism of defective viral RNA replication remains to be elucidated. Recent molecular genetic studies have identified a number of cis dominant mutations in 2B, 3A, 3C, and 5′NTR regions which caused defects in the enteroviral RNA synthesis [Hellen and Wimmer, 1995]. We consider that during cardiac viral replication, these CVB3-genomic regions involved in the positive-strand RNA replication could be modified by spontaneous dominant mutations in vivo. Using molecular cloning and direct sequencing methods, further investigations are in progress to identify viral specific mutations responsible for this defective RNA replication process. The determination of these nucleotidic sites are fundamental to the understanding of mechanisms by which CVB3 induce chronic myocarditis.

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